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Regulation of Ci and Su(fu) nuclear import in *Drosophila*

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Abstract

The Hedgehog (Hh) signal transduction pathway plays a central role in the development of invertebrates and vertebrates. While much is known about the pathway, the role of Suppressor of fused (Su(fu)), a component of the pathway's signaling complex has remained enigmatic. Previous studies have linked Su(fu) to the cytoplasmic sequestration of the zinc finger transcription factor, Cubitus interruptus (Ci), while other studies suggest a role in modulating target gene expression. In examining the cell biology of the pathway, we have found that like its vertebrate homologue, *Drosophila* Su(fu) enters the nucleus. Furthermore, we find that the nuclear import of Su(fu) occurs in concert with that of Ci in response to Hh signaling. Here, we examine the mechanism by which Su(fu) regulates Ci import by investigating the importance of the Ci nuclear localization signal (NLS) and the effect of adding an additional NLS. Finally, we demonstrate that Ci can bring Su(fu) with it to a multimerized Ci DNA binding site. These results provide a basis for understanding the dual roles played by Su(fu) in the regulation of Ci.

Keywords: *Cubitus interruptus*; *Suppressor of fused*; *Hedgehog*; Signal transduction; *Drosophila*

Introduction

Hedgehog (Hh) signal transduction plays a vital role in the normal development of both vertebrate and invertebrate tissues. The pathway functions to specify multiple cell fates in tissues by governing differential responses to the graded distribution of the secreted Hh molecule. These responses regulate cell proliferation and differentiation and are essential for the patterning of *Drosophila* embryonic segments and appendages (Hooper and Scott, 2005; Ingham and McMahon, 2001; Lum and Beachy, 2004).

In *Drosophila*, adult appendages are derived from larval imaginal discs. In the case of most discs including the wing, the tissue is separated into anterior and posterior compartments (Crick and Lawrence, 1975; Garcia-Bellido et al., 1973). Hh is produced and secreted by cells of the posterior compartment and received by anterior cells which are Hh responsive (Basler and Struhl, 1994). Those cells that receive the signal activate a signal transduction cascade which ultimately results in an alteration in transcriptional output. The relative distance of the receiving cells from the source of Hh modulates the transcriptional outcome of signal reception leading to at least two domains of gene expression (Hepker et al., 1997; Strigini and Cohen, 1997; Vervoort et al., 1999; Wang and Holmgren, 1999). Cells closest to Hh-secreting posterior cells respond by expressing several genes which require high-level signaling such as *patched* (*ptc*) and *engrailed* (*en*) (Blair, 1992). Anterior cells more distant from the source of Hh respond by expressing a different set of genes including *decapentaplegic* (*dpp*) (Methot and Basler, 1999; Strigini and Cohen, 1997).

Hh signal transduction in anterior cells is initiated at the plasma membrane where Hh interacts with its receptor, Patched (Ptc), a twelve pass transmembrane protein (Marigo et al., 1996; Stone et al., 1996) that functions to catalytically repress another transmembrane protein, Smoothened (Smo) in the absence of Hh (Alcedo et al., 1996; Taipale et al., 2002; van den Heuvel and Ingham, 1996).

Derepression of Smo leads to transmission of a signal from the surface of the cell to a cytoplasmic complex of several proteins which function to regulate Cubitus interruptus (Ci), the transcriptional mediator of the Hh signal (Alexandre et al., 1996; Hepker et al., 1997). These proteins include Suppressor

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of Fused (Su(fu)), a novel PEST-domain containing protein, Fused (Fu), a serine/threonine kinase, and Costal-2, a kinesin-like protein (Alves et al., 1998; Pham et al., 1995; Preat, 1992; Preat et al., 1990; Robbins et al., 1997; Sanchez-Herrero et al., 1996; Sisson et al., 1997). Recent evidence suggests that the signal may be transmitted directly from Smo to Cos2, as an interaction between the two proteins has been identified (Jia et al., 2003; Lum et al., 2003; Ogden et al., 2003; Ruel et al., 2003).

The proteins of the cytoplasmic complex function together to modulate the activity of Ci, which can act either as a transcriptional activator or repressor. In the absence of Hh, the repressor form of Ci (Ci-75) is generated by proteolytic processing of the full-length molecule to yield a C-terminally truncated protein that lacks a transcriptional activation domain but retains the ability to bind DNA via the zinc finger binding domain (Aza-Blanc et al., 1997). Conversely, the transcriptional activator Ci (Ci-155) has the ability to activate its targets (Alexandre et al., 1996; Hepker et al., 1997). A second method by which Ci is regulated is via "activation". At the anterior/posterior boundary, Ci-155 acts as a more potent transcription factor in response to the highest exposure to Hh (Methot and Basler, 1999; Price and Kalderon, 1999; Wang and Holmgren, 1999). Finally, Ci is regulated by a third mechanism, Hh-dependent nuclear import of the transcription factor (Chen et al., 1999; Wang et al., 2000; Wang and Holmgren, 2000).

The nuclear import of Ci-155 is primarily modulated by three components of the Hh signaling machinery, Cos2, Fu, and Su (fu), all of which act to oppose the nuclear import of full-length Ci (Chen et al., 1999; Lefers et al., 2001; Methot and Basler, 2000; Wang et al., 2000; Wang and Holmgren, 2000). Yeast two-hybrid analysis, coimmunoprecipitations, and GST pull-down experiments have shown that all of these proteins are in complexes with Ci (Monnier et al., 2002; Stegman et al., 2000). Biochemical data suggest that there are at least two different cytoplasmic complexes, a trimeric complex of Ci, Cos2 and Fu, which is associated with microtubules, and a tetrameric complex of Ci Cos2, Fu, and Su(fu) (Stegman et al., 2000).

In addition to its role in modulating the subcellular distribution of Ci, there is evidence that Su(fu) inhibits the action of Ci by a mechanism independent of cytoplasmic retention in *Drosophila* (Wang et al., 2000). Studies in mammals show that Su(fu) enters the nucleus with Gli1, and in vitro experiments demonstrate that Su(fu) enhances the ability of Gli proteins to bind their DNA targets. (Kogerman et al., 1999; Pearse et al., 1999). Furthermore, mammalian Su (fu) specifically interacts with SAP18, a component of the mSin3 and histone deacetylase complex and in tissue culture acts to repress transcription from Gli-dependent promoters (Cheng and Bishop, 2002; Paces-Fessy et al., 2004). This provides a mechanism by which Su(fu) could modulate the transcriptional potential of Ci. However, previously, there have been no reports of nuclear Su(fu) in *Drosophila*.

Here, we investigate the roles that Su(fu) plays in the regulation of Ci. We find that Su(fu), like its mammalian homologue, can translocate to the nucleus. Furthermore, the nuclear import of Su(fu) requires Ci and is regulated by Hh. We go on to examine how the nuclear import of Ci and Su(fu) is regulated and what role Su(fu) may play in the nucleus.

Materials and methods

Fly strains

4bs-lacZ (Hepker et al., 1999), *UAS-CiN[HA]Zn* (Hepker et al., 1997), *UAS-Su(fu)* (Croker et al., 2006), and *ci-GAL4* (Croker et al., 2006) have been described previously. The HA tag in *UAS-CiN[HA]Zn* was inserted into a PstI site and should not interfere with Su(fu) binding. *sgs3-GAL4* (Cherbas et al., 2003) was obtained from A. Andres. *yw Actin/CD2/GAL4* was obtained from L. Zipursky (Pignoni and Zipursky, 1997). *UAS-myc-Su(fu)* (Methot and Basler, 2000) was obtained from K. Basler. FRT42D*cos2*^{W1} was obtained from T. Orenic. *smo*^{D16} was obtained from G. Struhl. Other stocks were obtained through Bloomington Stock Center.

DNA constructs

All *ci* constructs were derived from the *pGEM7Zf-Ci-cDNA* construct described previously (Hepker et al., 1997). They were all cloned as BamHI-BglII fragments into *pUAST* (Brand and Perrimon, 1993) and integrated into the fly genome by germ line transformation. *UAS-Ci(X)* refers to any of the various constructs used in this study, including those described below. Multiple independent lines were established.

UAS-Ci-SV40NLS

The coding sequence of the SV40NLS (PKKKRKV) was inserted at the PstI site in full-length *ci* using two complementary oligos: 5′-CCTAAGAAGAAACGTAAGGTAGAATTCCCTGCA-3′ and 3′-ACGTG-GATTCTTCTTTGCATTCCATCTTAAGGG-5′. The insertion was sequence verified.

UAS-CimutNLS

Sequences between the NcoI (nucleotide 2221/2222) and HpaI (nucleotide 2469/2470) sites were deleted from the full-length *ci* and replaced with a NcoI-XbaI linker plus a XbaI-HpaI PCR fragment. The linker was created with the following two complementary oligos: 5′-CATGGAGCTGAATTTTATG-CAAATGGAGGT-3′ and 3′-CTCGACTTAAAATACGTTTACCTCCA-GATC-5′. The PCR primers used to generate the XbaI-HpaI fragment were 5′-GCTCTAGAGGGGTTGCCTCTAAATGAC-3′ and 3′-AACAAACTGT-CAGATGCTCGAACTCC-5′. The sequence of the linker including the new XbaI site corresponds to the substitution of amino acids KKHK with GGLE. The mutation was verified by sequencing.

300bs-lacZ

A fragment containing four Ci binding sites as described in Hepker et al. (1999) was multimerized into eighty tandem repeats and cloned into the Pelican vector (Barolo et al., 2000) as a XbaI-SalI fragment.

Experimental fly crosses

Ci wing imaginal disc nuclear import assay

Progeny from the following cross were heat shocked 24–48 h after egg laying for 60 min at 32°C to generate *UAS*-Ci(X) expressing clones:

yw Actin/CD2/GAL4 X ywHSFlp; UAS-Ci(X)
yw Actin/CD2/GAL4; UAS-Su(fu) X ywHSFlp; UAS-Ci(X)
yw Actin/CD2/GAL4; UAS-Ci(X) X ywHSFlp; UAS-Su(fu); 4bs-lacZ

Larvae were raised at 18°C to reduce GAL4 activity, and LMB treatment was performed on third instar wing imaginal discs as previously described (Fukuda et al., 1997; Wang and Holmgren, 2000). The efficacy of LMB treatments could be assessed by following endogenous Ci localization at the compartment boundary in response to high levels of Hh signaling which induces nuclear entry.

Transactivation assay

Progeny from the following cross were heat shocked 24–48 h after egg laying for 60 min at 35°C to generate *UAS*-Ci(X) expressing clones:

yw Actin/CD2/GAL4; UAS-Ci(X) X ywHSFlp; UAS-Su(fu); 4bs-lacZ

Generation of somatic clones

Clones of mutant cells were generated through FLP-mediated mitotic recombination (Xu and Rubin, 1993). *cos2* and *smo* loss-of-function clones were created 48–72 h after egg laying by heat shocking larvae from the following crosses for 60 min at 35°C and treating with LMB for 1 h (Fukuda et al., 1997):

yHSFlp; FRT42myc45 X FRT42Dcos^{W1}/CyO

smo loss-of-function clones were created as indicated above from the following cross:

yw; smo^{D16} FRT40A/CyO X ywHSFlp; ubi-GFP FRT40A/CyO

Salivary gland nuclear import assay

Mid third instar larval salivary glands were dissected from the following crosses:

sgs3-GAL4 X UAS-Ci(X)
sgs3-GAL4 X UAS-Su(fu)
sgs3-GAL4; UAS-myc-Su(fu) X UAS-Ci(X)
sgs3-GAL4 X UAS-Ci, UAS-Su(fu)
UAS-Su(fu); sgs3-GAL4 X UAS-Ci(X)

Dissected glands were placed in S2 media at pH 7.2 containing LMB as previously described (Fukuda et al., 1997; Wang and Holmgren, 2000). The time courses were conducted by incubating the glands in LMB as described above for 30 min or 2 h, followed immediately by fixation.

Cuticle assays

The ability of the various *UAS-Ci* constructs to rescue *ci* mutant embryos was tested as follows:

yw; ci-GAL4/CyO*; ci^{94}/y^{+}* X *yw; UAS-Ci(X)/+; ci^{94}/y^{+}*

Cuticle mounts were as described (van der Meer, 1977). Embryos homozygous mutants for the endogenous *ci* gene were recognized by their yellow denticle belts. For *UAS-Ci-SV40NLS*, one line was analyzed, and for *UAS-CimutNLS*, two independent lines were analyzed. For wild-type *UAS-Ci*, nine independent lines were analyzed, and all gave a wild-type phenotype.

Immunohistochemistry

Imaginal discs were prepared as in Carroll and Whyte (1989). Salivary glands were prepared in a similar manner. Images were collected on a Zeiss Axiophot Microscope fitted with a digital camera and analyzed with VayTek deconvolution software. Antibody stainings of imaginal discs and salivary glands were performed with the monoclonal antibody 2A1 (Motzny and Holmgren, 1995), which only recognizes the full-length form of Ci, anti-β-Galactosidase (Zhang et al., 1994), anti-HA (Abcam, Cambridge, UK), anti-GFP (Molecular Probes, Eugene, OR), or anti-Su(fu) (Lum et al., 2003) (Developmental Studies Hybridoma Bank). All secondary antibodies were purchased from Jackson Immunoresearch Laboratory. DAPI was obtained from Sigma.

Results

Su(fu) enters the nucleus with Ci in a Hh-dependent manner

In order to address cell biological issues, we have begun utilizing the large cells of salivary glands as a model system to study certain aspects of Hh signal transduction. Individual

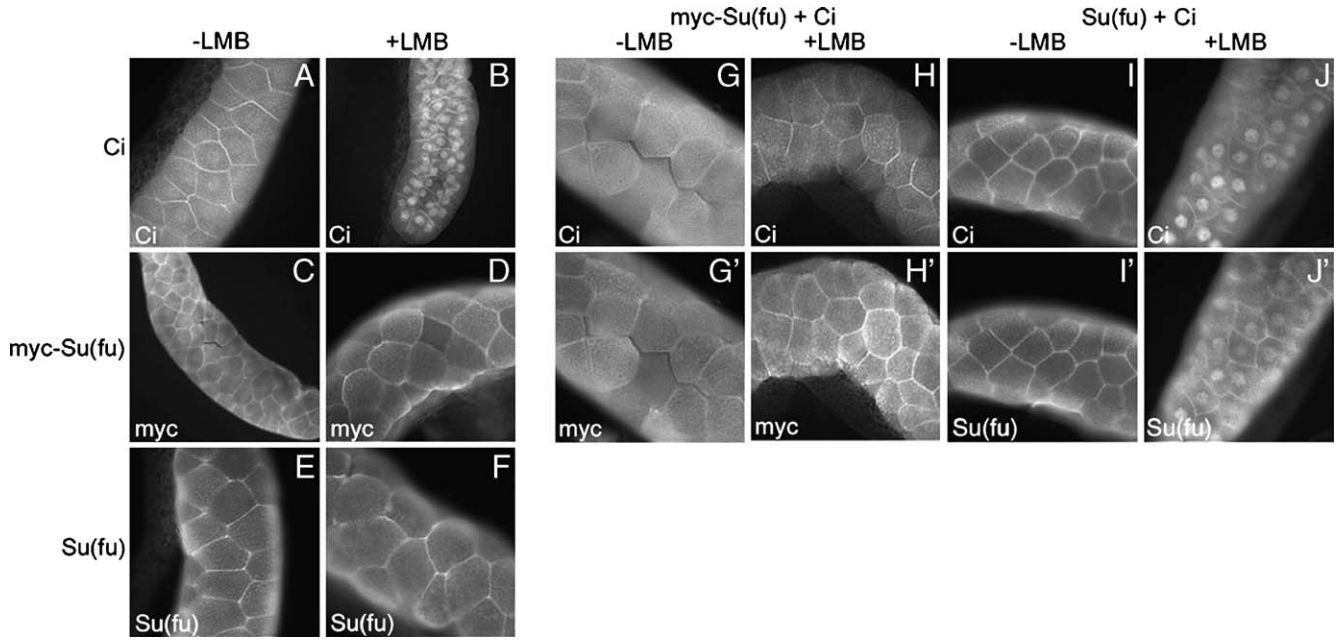

Fig. 1. Nuclear import assay of salivary glands expressing *UAS-Ci*, *UAS-myc-Su(fu)*, or *UAS-Su(fu)* individually or in combination. Glands overexpressing *UAS-Ci* treated with (B) and without LMB (A). (B) Ci localizes to the nucleus following LMB treatment. (C–D) Glands overexpressing *UAS-myc-Su(fu)* treated with (D) and without LMB (C). (E–F) Glands overexpressing *UAS-Su(fu)* treated with (F) and without LMB (E). When expressed on their own *UAS-myc-Su(fu)* and *UAS-Su(fu)* are cytoplasmic (C–F). (G–H) Glands overexpressing *UAS-Ci* and *UAS-myc-Su(fu)* treated with (H, H′) and without LMB (G, G′). N-terminally tagged Su(fu) displays a mutant phenotype and sequesters Ci in the cytoplasm (H). (I–J) Glands overexpressing *UAS-Ci* in combination with *UAS-Su(fu)* treated with (J, J′) and without LMB (I, I′). In the absence of other components of the Hh signal transduction cascade Su(fu) and Ci enter the nucleus. All UAS constructs were driven by *sgs3*-GAL4.

components of the pathway were overexpressed using the salivary gland specific driver *sgs3Gal4* and their subcellular distributions visualized in the presence or absence of leptomycin B (LMB), a drug which blocks Crm1 mediated nuclear export (Fukuda et al., 1997) (Figs. 1A–F). Previous work has shown that Ci has both nuclear import and nuclear export signals. Therefore, to assay Ci nuclear import, it is necessary to block nuclear export to prevent the shuttling of Ci in and out of the nucleus. In the absence of LMB, Ci is primarily cytoplasmic (Fig. 1A). While it appears that much of the Ci protein is localized to the plasma membrane, this pattern depends upon the age of the salivary glands. In younger salivary glands, Ci is more uniformly distributed (data not shown). The shift in Ci distribution correlates with a corresponding change in the disposition of microtubules, which also change from being relatively uniform in young glands to concentrating adjacent to the plasma membrane in older salivary glands (Riparbelli et al., 1993). The distribution of Ci dramatically changes in the presence of LMB, as the majority of Ci is now trapped in the nucleus (Fig. 1B).

The next experiment was to determine the subcellular distribution of Su(fu). Initial overexpression studies with Su(fu) had been carried out using an N-terminal myc tag (Methot and Basler, 2000). Recently, a monoclonal antibody to Su(fu) was produced (Lum et al., 2003) enabling untagged Su(fu) to be visualized. The distribution of Su(fu) and myc-Su(fu) was analyzed both in the presence and absence of LMB. Under both conditions, myc-Su(fu) is distributed throughout the cytoplasm (Figs. 1C–D). Similarly, untagged Su(fu) was also found to localize to the cytoplasm in the absence or presence of LMB (Figs. 1E and F, respectively), suggesting that on their own, Su(fu) and myc-Su(fu) appear to remain in the cytoplasm.

After analyzing the nuclear import of the individual proteins within salivary glands, the ability of myc-Su(fu) and Su(fu) to sequester Ci in the cytoplasm was assayed. In the presence or absence of LMB, glands overexpressing myc-Su(fu) and Ci exhibit a cytoplasmic distribution of both myc-Su(fu) (Figs. 1G′ and H′) and Ci (Figs. 1G and H). This is in contrast to the result in glands that overexpress Ci and untagged Su(fu) (Figs. 1I–J). In the presence of LMB, both Ci and Su(fu) readily enter the nucleus, and only a small portion of Ci remains in the cytoplasm with Su(fu) (Figs. 1J and J′). The presence of the N-terminal myc tag on Su(fu) appears to prevent the Ci-Su(fu) complex from entering the nucleus. Interestingly, a C-terminally tagged Su(fu)-YFP construct also appears to be defective, as Su(fu)-YFP fails to enter the nucleus with Ci after treatment with LMB (data not shown).

Given that previous studies examining the subcellular distribution of Su(fu) had been done with tagged constructs, it seemed appropriate to revisit this question. The nuclear import of endogenous Ci and Su(fu) was analyzed in wing imaginal discs. In response to Hh signaling along the anterior/posterior (A/P) compartment border, Ci shuttles in and out of the nucleus. This can be observed by blocking nuclear export with LMB. In

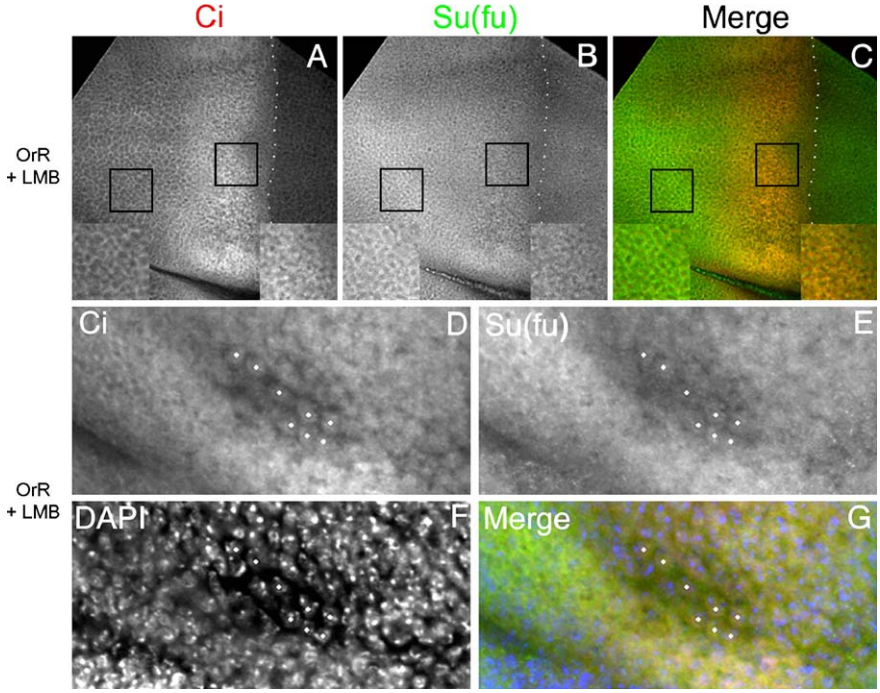

Fig. 2. *Drosophila* Su(fu) enters the nucleus in response to Hh. In all panels: anterior is to the left, dorsal is at the top, and where applicable, the A/P boundary is delineated by a dotted line. (A–G) Wing imaginal discs treated with LMB to block nuclear export. (A) Endogenous Ci is nuclear at the A/P boundary in the presence of Hh. (B) Endogenous Su(fu) staining shows expression in both compartments. At the A/P boundary, Su(fu) is nuclear in response to Hh. (C) Merge of panels A and B. Insets highlight the nuclear staining of Su(fu) and Ci at the A/P boundary and the honeycomb like pattern indicates cytoplasmic Ci and Su(fu) away from the boundary. The nuclear accumulation of Ci and Su(fu) is especially evident at the fold in the wing disc where cells are more sparsely positioned (D and E, respectively), and colocalization with DAPI (F) confirms accumulation in the nucleus (G). (G) In the merged image, Ci staining is in red, Su(fu) staining in green and DAPI staining in blue. Several nuclei that have accumulations of Ci and Su(fu) are indicated by white dots.

wing imaginal discs, both Ci and Su(fu) enter the nucleus in the anterior compartment along the A/P boundary (Figs. 2A and B, respectively). The colocalization of Ci and Su(fu) is especially evident near folds in the wing imaginal disc where cells are more sparse (Figs. 2D and E, respectively) and overlap with DAPI staining confirms the nuclear accumulation of these two proteins (Fig. 2G). Away from the boundary in the anterior compartment, Ci and Su(fu) are cytoplasmic, and in the posterior compartment, Su(fu) is also cytoplasmic (Figs. 2A–C). These results suggest that nuclear import of Su(fu) is dependent upon both Ci and Hh signaling. Directly adjacent to the A/P boundary where the level of Hh signaling is highest, it has been previously observed that Ci protein levels are attenuated, and the same is true for Su(fu) (Figs. 2A–B).

N-terminally myc-tagged Su(fu) is tethered in the cytoplasm

Two possible explanations for the sequestration of myc-Su(fu) and Ci in the cytoplasm are myc-Su(fu) masking the NLS of Ci or myc-Su(fu) causing the proteins to be bound to some component anchored in the cytoplasm. To address this question, we examined the effects of either mutating the endogenous Ci NLS or adding a SV40 NLS to Ci by assaying how these modified proteins interact with Su(fu) and myc-Su(fu).

The rate of Ci nuclear entry in salivary glands was examined by following the subcellular distribution of Ci after 30 min or 2 h of LMB treatment. Thirty minutes after the addition of LMB wild-type Ci begins to accumulate in the nucleus of salivary glands (Fig. 3A), and after 2 h of LMB treatment, Ci localizes

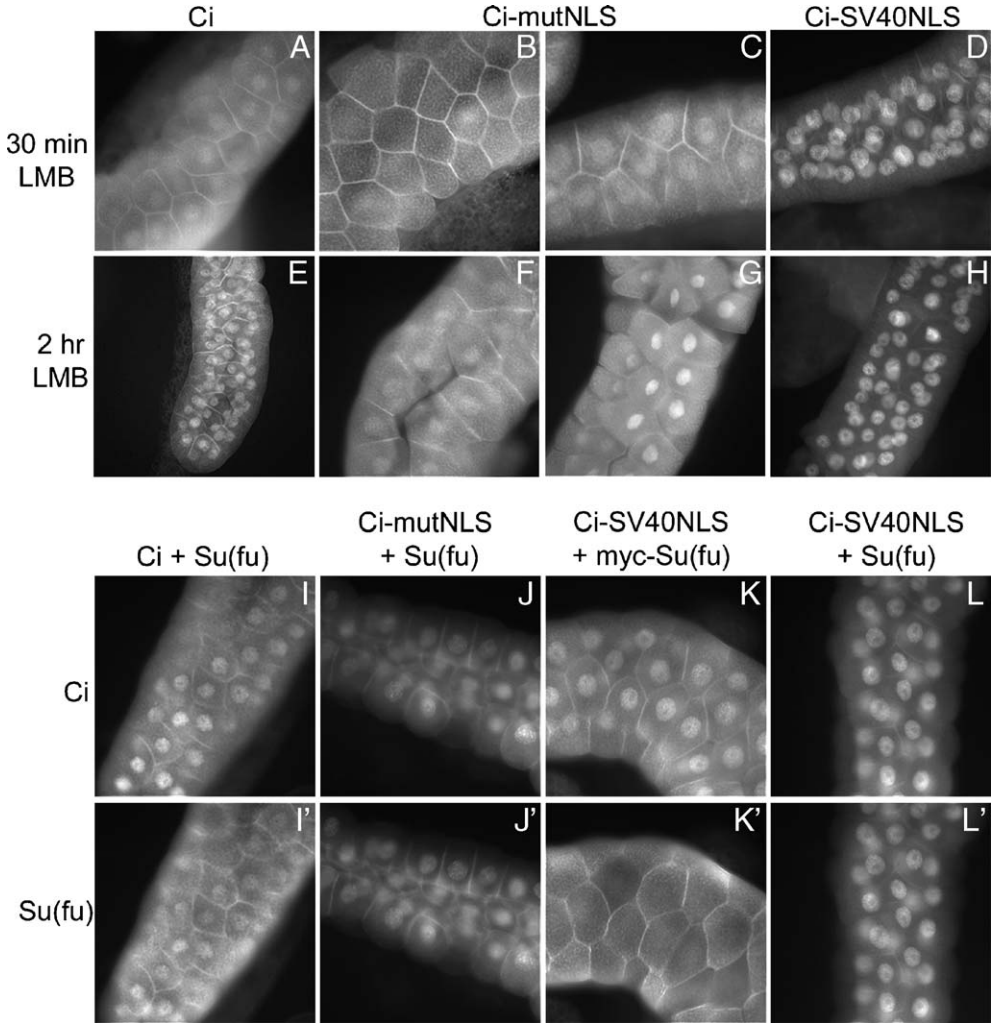

Fig. 3. myc-Su(fu) prevents Ci nuclear entry by cytoplasmic tethering. Time course of nuclear import of Ci in salivary glands (A–H). (A–D) Mid-third instar salivary glands treated with LMB for 30 min to block nuclear export. (E–H) Glands treated with LMB for 2 h to block nuclear export. (A) Gland expressing *UAS-Ci*. After 30 min of LMB treatment, there is a slight accumulation of Ci in the nucleus. As expected, the nuclear accumulation of Ci is dramatically increased after 2 h of LMB treatment (E). (B, C, F, G) A range of phenotypes is observed in glands expressing *UAS-CimutNLS* in which the NLS has been mutated and that have been treated with LMB for 30 min (B, C) or for 2 h (F, G). Mutating the NLS appears to have a modest effect on the rate of nuclear entry of Ci. (D, H) Glands expressing *UAS-Ci-SV40NLS*, a Ci construct with the NLS signal from SV40 added to the N terminus. (D) 30-min LMB treatment of *UAS-Ci-SV40NLS*. (H) 2-h LMB treatment of *UAS-Ci-SV40NLS*. With the addition of the SV40 NLS Ci is trapped in the nucleus within 30 min. (I–L) Two-hour LMB treatments of mid-third instar glands expressing *UAS-Ci* and *UAS-Su(fu)*, *UAS-CimutNLS* and *UAS-Su(fu)*, *UAS-Ci-SV40NLS* and *UAS-myc-Su(fu)*, or *UAS-Ci-SV40NLS* and *UAS-Su(fu)*. (I) Gland expressing *UAS-Ci* and *UAS-Su(fu)* stained for Ci (I) or for Su(fu) (I′). Gland expressing *UAS-CimutNLS* and *UAS-Su(fu)* stained for Ci (J) or for Su(fu) (J′). Gland expressing *UAS-Ci-SV40NLS* and *UAS-myc-Su(fu)* stained for Ci (K) and myc (K′). Gland expressing *UAS-Ci-SV40NLS* and *UAS-Su(fu)* stained for Ci (L) and Su(fu) (L′). All UAS constructs were driven by *sgs3*-Gal4. Whereas Ci-SV40NLS readily brings wild-type Su(fu) with it into the nucleus, myc-Su(fu) remains tethered in the cytoplasm.

primarily in the nucleus (Fig. 3E). After 30 min of LMB treatment, Ci-mutNLS, a construct in which the NLS of Ci is mutated, showed distributions of Ci that ranged from a few cells to every cell exhibiting a slight nuclear accumulation of Ci-mutNLS (Figs. 3B and C, respectively). After 2 h of LMB treatment, some glands only had a slight nuclear accumulation (Fig. 3F); however, many glands had a large amount of nuclear Ci-mutNLS (Fig. 3G). This suggests that mutating the NLS may delay but does not block the nuclear import of Ci. Finally, a construct in which the NLS from viral SV40 was added near the N terminus of Ci, Ci-SV40NLS, showed strong nuclear accumulation after only 30 min of LMB treatment (Figs. 3D and H). Similar to previous studies (Wang and Jiang, 2004), the addition of an SV40 NLS results in rapid Ci nuclear import.

We next examined whether wild type Su(fu) would sequester Ci-mutNLS in the cytoplasm. Glands coexpressing *UAS-Ci-mutNLS* and *UAS-Su(fu)* were treated with LMB for 2 h and stained for Ci and Su(fu) respectively (Figs. 3J and J′). Mutating the NLS appears to have little effect on the overall import of Ci-mutNLS or Su(fu), as the protein distribution is quite similar to the control of Ci and Su(fu) following LMB treatment (Figs. 3I and I′). N-terminally myc-tagged Su(fu) was then coexpressed with Ci-SV40NLS to determine if Ci-SV40NLS would bring myc-Su(fu) into the nucleus. After the 2-h LMB treatment, Ci-SV40NLS was primarily nuclear (Fig. 3K); however, relative to Ci-SV40NLS expressed on its own, a significant fraction of the protein remained sequestered in the cytoplasm, presumably by myc-Su(fu). N-terminally myc-tagged Su(fu), however, remained cytoplasmic and appeared not to enter the nucleus (Fig. 3K′). These results were dramatically different than LMB treated glands expressing *UAS-Ci-SV40NLS* and *UAS-Su(fu)*. In this case, both Ci-SV40NLS and Su(fu) were strongly nuclear (Figs. 3L and L′, respectively). Taken together, these results show Ci-SV40NLS has the ability to efficiently bring wild-type Su(fu) with it into the nucleus, but that it is incapable of removing myc-Su(fu) from its cytoplasmic tether.

We decided to extend these experiments by examining the consequences of removing or adding an NLS to Ci in the embryonic epidermis and in imaginal discs where the cells are Hh responsive.

Loss and addition of an NLS to Ci have opposite effects on epidermal patterning in the embryo

To determine the requirement of the endogenous Ci NLS and the effect of adding an exogenous NLS, we analyzed the ventral abdominal denticle pattern of larval cuticles in which endogenous Ci was replaced by the expression of various modified Ci constructs. The ci^{94} null background has a very characteristic ventral phenotype in which the regions of naked cuticle are replaced by mirror-image duplications of denticles (Fig. 4A) (Slusarski et al., 1995). We used *ci-GAL4* to express *UAS-Ci* constructs in the endogenous *ci* expression pattern. ci^{94} null mutant animals were identified by their yellow denticle belts. Mutant animals expressing a *UAS-Ci* construct were recognized by a change in denticle belt patterning that was distinct from controls.

Expression of *UAS-Ci-cDNA* in ci^{94} mutants (Fig. 4B) completely restores the wild-type repetitive pattern of denticles and naked cuticle in embryos. These animals complete development and eclose into normal-looking, sterile adults. Expression of *UAS-CimutNLS* in ci^{94} mutants results in a loss of naked cuticle between some denticle belts (Fig. 4C). This phenotype is indicative of inappropriate downregulation of the

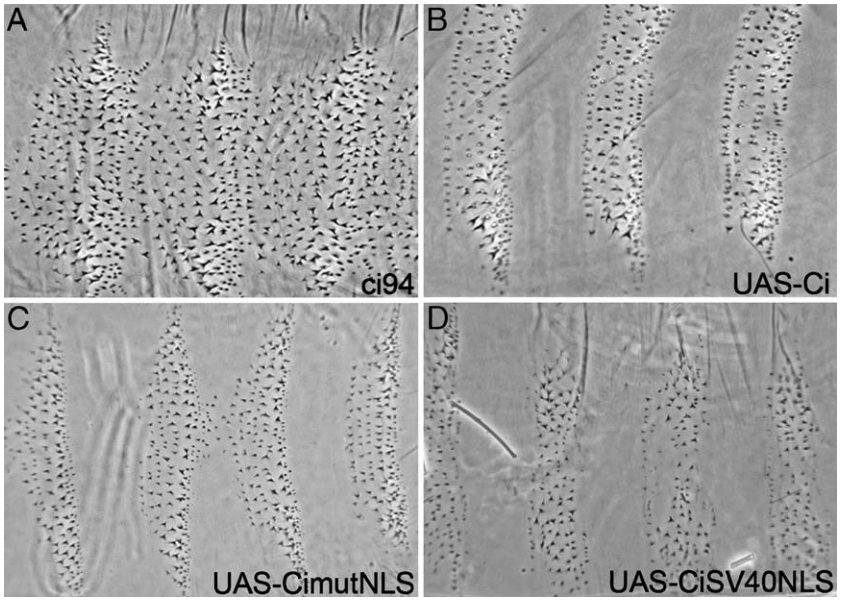

Fig. 4. Cuticle mounts were prepared of ci^{94} embryos (A) in which various *UAS* constructs were expressed using *ci-GAL4* (A-D 25× images). (B) Expression of *UAS-Ci-cDNA* can fully rescue a null mutant phenotype, restoring regions of naked cuticle and denticle patterning. (C) Deletion of the endogenous NLS region leads to a slight loss of naked cuticle, consistent with evidence indicating an impediment to nuclear entry of the molecule. (D) Expression of *UAS-Ci-SV40NLS* generates a variable phenotype ranging from a complete loss of the denticles to deletion of portions of the abdominal denticle belts (the most mild phenotype is shown in panel D). This result is consistent with deregulated nuclear accumulation of this molecule.

Hh signal, as formation of naked cuticle is the result of Hh maintenance of *wingless* expression. This result is consistent with our data which indicate that the nuclear import of CimutNLS may be less efficient when compared to wild type. Expression of *UAS-Ci-SV40NLS* in ci^{94} mutants results in a range of phenotypes from a complete loss of the denticle belts to a reduction in the number of denticles and a change in the overall shape of the denticle belts. Shown in Fig. 4D is an example of a mild phenotype in which the denticle belts are not fully formed and are missing groups of denticles. This phenotype is indicative of ectopic Hh signaling and suggests that addition of an exogenous NLS generates a somewhat hypermorphic or gain-of-function molecule.

The endogenous Ci NLS is not required for appropriate nuclear import in wing discs and the addition of an SV40 NLS leads to Hh independent nuclear import of Ci and Su(fu)

Clones expressing various *UAS-Ci* proteins were generated in wing imaginal discs using an *Actin5Cp*-"flip-out"-*GAL4* driver (Pignoni and Zipursky, 1997) to express moderate levels of the *UAS* constructs. Discs were subsequently treated with LMB, and the subcellular distribution of Ci was assayed. In anterior compartment clones away from the A/P boundary, wild-type Ci expressed at moderate levels did not accumulate in the nucleus, whereas wild-type Ci in posterior compartment clones and endogenous Ci along the A/P boundary were nuclear (Fig. 5A). The subcellular distribution of clonally expressed *UAS-CimutNLS* was identical to that of moderately expressed wild type Ci (Fig. 5C). This result suggests that in the context of the wing disc, the endogenous NLS of Ci is dispensable in terms of appropriate subcellular distribution of the molecule. Conversely, clonally expressed *UAS-Ci-SV40NLS* as well as a highly expressed wild-type Ci construct were observed to accumulate in the nucleus in the presence of LMB regardless of clone location (Figs. 5D and B, respectively).

As the level of endogenous Su(fu) may be limiting and previous studies have shown that Su(fu) is capable of sequestering overexpressed wild-type Ci in the cytoplasm

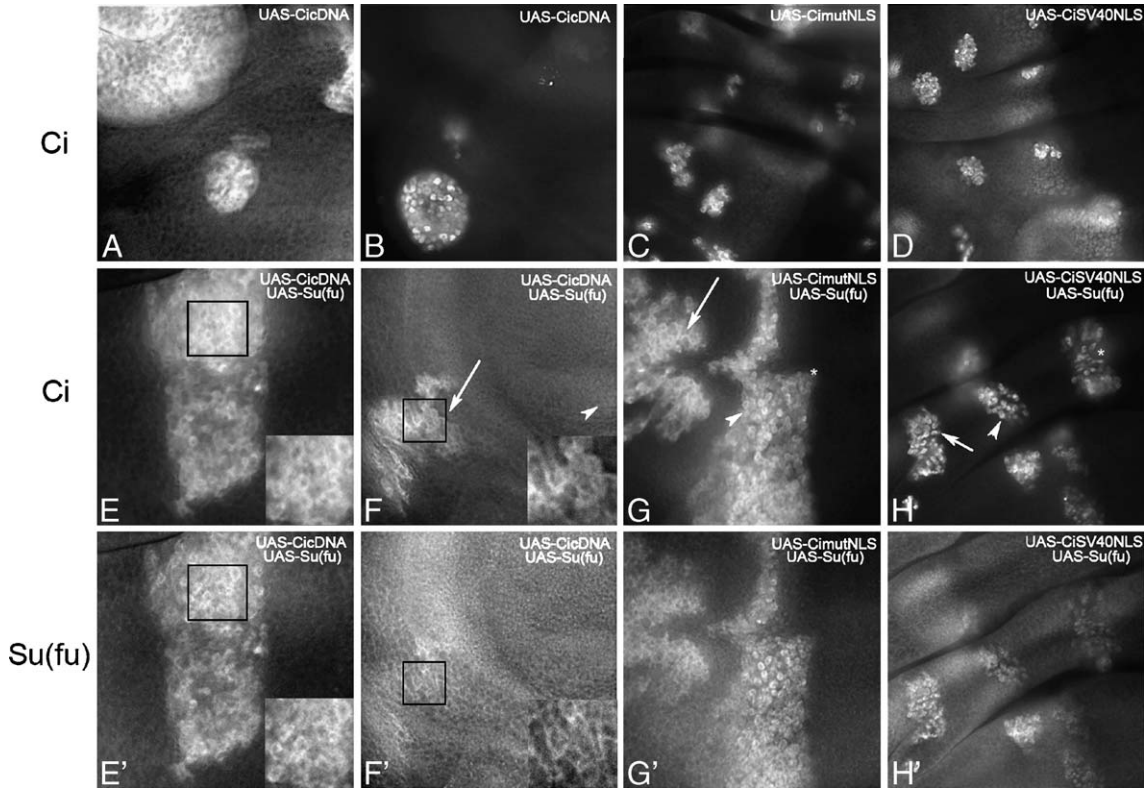

Fig. 5. The subcellular distribution of *UAS-Ci* constructs was assayed in the anterior compartment of wing imaginal discs (dorsal is up, anterior is to the left). All constructs were induced clonally using an Actin5Cp-"flip-out"-*GAL4* driver. Nuclear export was inhibited in wing discs with LMB. Arrowheads indicate nuclear accumulation of Ci along the A/P boundary in panels F–H (boundary region not shown in A and E), arrows indicate clones within the anterior compartment but removed from Hh signaling, and asterisks indicate posterior clones which receive high levels of Hh signaling. (A) Despite overexpression in the anterior compartment, clonally expressed Ci from a modestly expressed *UAS-Ci-cDNA* does not accumulate in the nucleus. Clonally expressed Ci from a highly expressed *UAS-Ci-cDNA* does accumulate in the nucleus in the anterior compartment (B), as does *UAS-Ci-SV40NLS* (D). Conversely, *UAS-CimutNLS* localizes to the cytoplasm in anterior clones and accumulates in the nucleus along the A/P boundary, suggesting that nuclear import of this molecule is not dependent on the presence of the endogenous NLS (C). Coexpression of *UAS-Su(fu)* leads to a redistribution of the highly expressed *UAS-Ci-cDNA* in the anterior compartment to the cytoplasm while having no effect on the nuclear localization or *UAS-Ci-SV40NLS* in the same compartment (F and H, respectively). As expected, coexpression of *UAS-Su(fu)* has no effect on the cytoplasmic distribution of the more moderately expressed *UAS-Ci-cDNA* or *UAS-CimutNLS* in the anterior compartment (E and G, respectively). In the presence of high levels of Hh either at the compartment boundary or in the posterior compartment, excess Su(fu) is not able to titrate Ci into the cytoplasm regardless of expression level or modification of the *UAS-Ci* (F, G, and H) and Su(fu) always colocalizes with Ci (F′, G′ and H′).

(Wang et al., 2000), we coexpressed *UAS-Su(fu)* with the Ci constructs and assayed the subcellular distribution of Ci. We exploited a highly expressed wild-type *UAS-CicDNA* which localizes to the nucleus away from high level Hh signaling (Fig. 5B). Addition of Su(fu) led to a shift in the distribution of wild-type Ci such that it accumulated in the cytoplasm away from Hh (Fig. 5F). Conversely, overexpression of Su(fu) did not alter the nuclear accumulation of *UAS-Ci-SV40NLS*, suggesting that addition of an exogenous NLS to Ci is sufficient to overcome the ability of Su(fu) to sequester Ci in the cytoplasm (Fig. 5H). Furthermore, Su(fu) also localizes to the nucleus in this context due to its interaction with the Ci molecule (Fig. 5H′). As expected, overexpression of Su(fu) with moderately expressed Ci or *UAS-CimutNLS* had no effect on the cytoplasmic distribution of Ci (Figs. 5E and G, respectively). Finally, overexpressed Su(fu) in the context of high level Hh signaling (either in the posterior compartment or along the A/P boundary) is not able to sequester excess Ci of any type into the cytoplasm, even when the NLS of Ci is mutated. These data suggest that Hh induces nuclear entry of the Su(fu)/Ci complex, and that this nuclear entry occurs independent of the function of the endogenous Ci NLS.

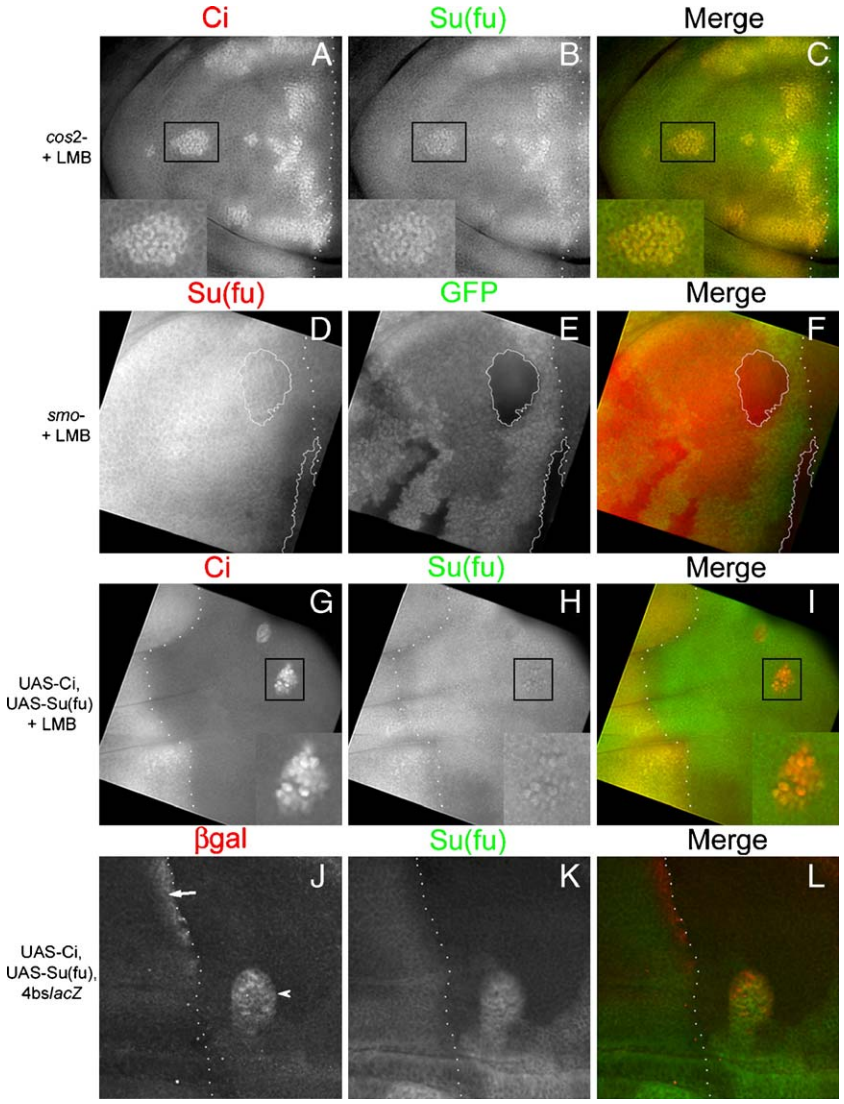

Fig. 6. In all panels: anterior is to the left, dorsal is at the top, and the A/P boundary is delineated by a dotted line. (A–I) Wing imaginal discs treated with LMB to block nuclear export. (A–C) Anterior compartment *cos2* loss-of-function clones treated with LMB and stained for Ci (A) and Su(fu) (B). In the absence of *cos2*, Ci and Su (fu) are nuclear. (C) Merge of panels A and B. Clones were identified by the ectopic expression of Ci. (D–F) Nuclear localization of Su(fu) is Hh dependent. Anterior compartment *smo* loss-of-function clones treated with LMB and stained for Su(fu) (D) and GFP (E). Clones were identified by the loss of GFP. Su(fu) is cytoplasmic in clones lacking *smo* at both high and moderate levels of Hh indicated by the outlined clones. The compartment boundary was identified by nuclear Su(fu) and the morphology of the clones. (F) Merge of panels D and E. (G–I) Posterior "flip out" clones expressing *UAS-Ci* and *UAS-Su(fu)* are exposed to the highest levels of Hh and are nuclear following LMB treatment. Posterior clone stained for Ci (G) and for Su(fu) (H). Both Su(fu) and Ci localize to the nucleus in response to this high level of Hh. (I) Merge of panels G and H. (J–L) Su(fu) does not prevent activation of a reporter construct at high levels of Hh. *4bs-lacZ* reporter gene activation was assayed in posterior "flip out" clones of *UAS-Ci* and *UAS-Su(fu)*, which were stained for β-gal (J) and Su(fu) (K). The arrow indicates normal *4bs-lacZ* activation at the compartment boundary in response to high levels of Hh and the arrowhead indicates ectopic *4bs-lacZ* activation in a posterior Ci and Su(fu) overexpressing clone (J). (L) Merge of panels J and K.

The proper subcellular distribution of Ci and Su(fu) requires Cos2 and Smo

It has been previously shown that the Cos2 protein is required to sequester Ci in the cytoplasm in the absence of Hh signaling (Wang and Holmgren, 2000). To determine the effect of Cos2 on Su(fu), *cos2* mutant clones were generated and the subcellular distribution of Ci and Su(fu) assayed following treatment with LMB. As can be seen in Figs. 6A and B, both Ci and Su(fu) accumulate in the nucleus of *cos2* mutant clones in anterior compartment clones away from Hh signaling. *cos2* mutant clones were identified by an upregulation of Ci levels. Su(fu) protein levels concurrently increase in these clones. To further elucidate the Hh dependence of nuclear Su(fu), *smo* loss-of-function clones were generated in the anterior compartment at the A/P boundary (Figs. 6D–F). In the absence of *smo*, Su(fu) remains in the cytoplasm despite the presence of high levels of Hh (Fig. 6D).

Nuclear Su(fu) does not prevent activation of a reporter construct that requires high level Hh signaling

When exposed to the highest level of Hh at the A/P boundary, Ci acts as a more potent transcription factor, termed "activated Ci" (Methot and Basler, 1999; Wang and Holmgren, 1999). The nature of activated Ci is not known, but Su(fu) has been implicated in its regulation. One suggestion is that at intermediate levels of Hh, Ci and Su(fu) enter the nucleus together, but at high levels of Hh, when Ci is in its "active" state, Su(fu) dissociates from Ci and Ci enters the nucleus alone (Ingham and McMahon, 2001). In principle, one could follow the subcellular distribution of Su(fu) in the region directly adjacent to the compartment boundary where Hh signaling is highest, but because of the attenuated levels of Su(fu) and Ci in this region, it is very difficult to distinguish signal from background. As an alternative test to this hypothesis, posterior compartment clones expressing *UAS-Ci* and *UAS-Su(fu)* were created with an *Actin5Cp*-"flip-out"-*GAL4* driver (Pignoni and Zipursky, 1997) and subsequently treated with LMB. In the absence of Hh, LMB treated anterior clones expressing Ci and Su(fu) exhibit a cytoplasmic distribution (Figs. 5E, and E′, respectively). In posterior clones, however, Hh levels are high, and Ci and Su(fu) enter the nucleus in the presence of LMB (Figs. 6G–I). This suggests that both Ci and Su(fu) enter the nucleus even at the highest levels of Hh signaling. Furthermore, to verify that the presence of nuclear Su(fu) does not preclude the activation of target genes that require the highest levels of Hh, *GAL4* "flip-out" clones driving *UAS-Ci* and *UAS-Su(fu)* were assayed for activation of the reporter gene *4bs-lacZ* (Hepker et al., 1999). *4bs-lacZ* requires high level Hh signaling for activation, and indeed, it is expressed in posterior clones expressing Ci and Su(fu) (Figs. 6J–L).

Ci and Su(fu) bind polytene chromosome bands

In mammalian cells, Su(fu) enters the nucleus and the binding of Su(fu) to Gli1 enhances Gli1 binding to a target site oligo (Cheng and Bishop, 2002; Pearse et al., 1999).

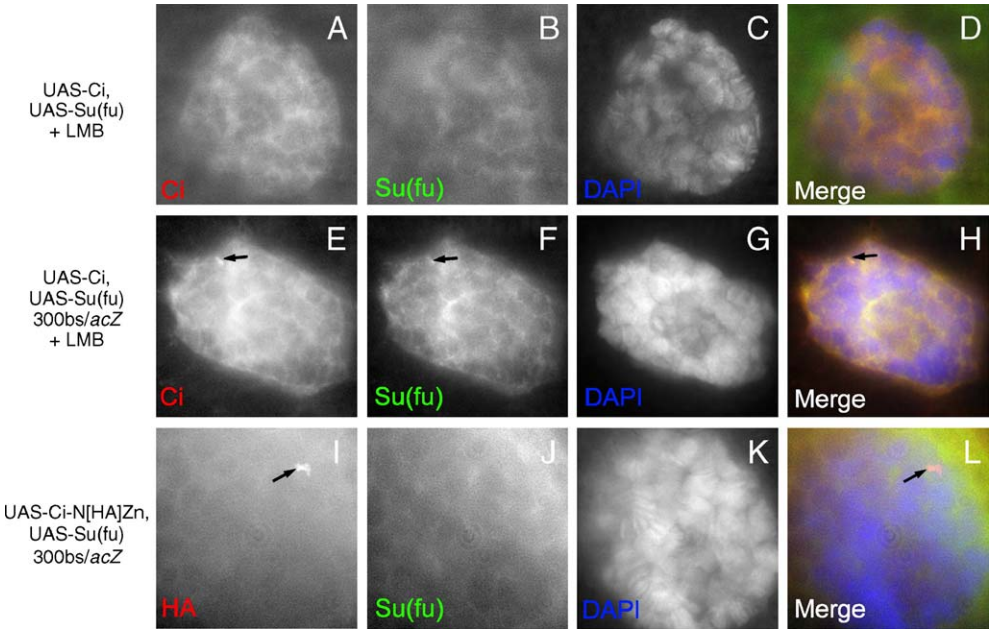

Fig. 7. Full-length Ci and Su(fu) bind a multimerized target on polytene chromosomes. (A–D) LMB treated salivary glands overexpressing *UAS-Su(fu)* and *UAS-Ci* were stained for Ci (A), Su(fu) (B), and DAPI (C). (D) Merge of panels A–C. Ci (A) and Su(fu) (B) are both nuclear but no specific staining is found on the polytene chromosomes Rather, Ci and Su(fu) are found in a fibrous pattern around the chromosomes. (E–H) A salivary gland that carries the *300bslacZ* target and overexpressing *UAS-Ci* and *UAS-Su(fu)* was stained for Ci (E), Su(fu) (F), and DAPI (G). (H) Merge of panels E–G. Arrows indicate the distinct bands formed from the binding of Ci and Su(fu) to the 300bs*lacZ* target on polytene chromosomes. (I–L) Su(fu) does not bind polytenes in the presence of CiN[HA]Zn. Salivary gland that carries the *300bslacZ* target and overexpressing *UAS-CiN[HA]Zn* and *UAS-Su(fu)* was stained for HA (I), Su(fu) (J), and DAPI (K). Arrows indicate the binding of CiN[HA]Zn to the *300bslacZ* target on polytene chromosomes. All UAS constructs were driven by *sgs3*-Gal4.

Given the conservation of the pathway, it might be expected that in *Drosophila* Ci would be able to bring Su(fu) to target enhancers. To investigate Su(fu) and Ci binding to DNA in the nucleus, we utilized the large cells of the *Drosophila* salivary glands to observe possible binding to polytene chromosomes. Aza-Blanc et al. (1997) first showed that the Ci-75 repressor binds polytene chromosome bands on chromosome squashes. Ci-75 binding can also be observed at a few sites on polytene chromosomes in salivary gland nuclei (unpublished observation), but labeling is not consistent, and it is not possible to determine the location of the binding sites. In the case of full-length Ci plus Su(fu), they are abundant in the nucleus following LMB treatment, but we have not observed binding to specific polytene chromosome bands in salivary gland nuclei (Figs. 7A–D). Therefore, to easily visualize the binding of full-length Ci and Su(fu) to a specific target in salivary gland nuclei, a construct containing 300 Ci binding sites, termed *300bs-lacZ*, was transformed into *Drosophila*. Salivary glands containing *300bs-lacZ* and expressing *UAS-Su(fu)* and *UAS-Ci* were treated with LMB, and the binding of Ci and Su(fu) to the target was assayed by antibody labeling. Binding of Ci and Su(fu) appears as a distinct band, which colocalize to the DAPI stained polytenes (Figs. 7E–H). When *UAS-Su(fu)* was overexpressed with *UAS-CiN[HA]Zn*, a construct containing the N terminus and Zn fingers of Ci, in glands containing *300bs-lacZ*, a distinct band was found on the polytenes indicative of Ci-N[HA]Zn binding, however, *UAS-Su(fu)* remained in the cytoplasm (Figs. 7I–L). The inability of Su(fu) to bind polytenes with Ci-N[HA]Zn is not surprising as it correlates with the loss of the C terminal Su(fu) binding domain within Ci (Croker et al., 2006) and is consistent with results showing that both the N and C terminal Su(fu) binding domains of Gli1 are required for Su(fu) cytoplasmic tethering of Gli1 (Merchant et al., 2004).

Discussion

Ci and Su(fu) enter the nucleus in response to Hh signaling

In this study, we establish the Hh dependent translocation of a *Drosophila* Su(fu)-Ci complex into the nucleus, illustrating further conservation between the mammalian and *Drosophila* Hedgehog signaling pathways. This result is somewhat of a paradox as Su(fu) has been shown to assist in the sequestration of Ci in the cytoplasm (Lefers et al., 2001; Methot and Basler, 2000; Wang et al., 2000; Wang and Holmgren, 2000). If Su(fu) contributes to the cytoplasmic sequestration of Ci, what is the mechanism that allows its release in response to Hh signaling? The likely event is phosphorylation by the Fu kinase (Preat et al., 1990). In the absence of Fu kinase function, Ci is not released in response to Hh signaling (Wang and Holmgren, 2000). However, in double mutants lacking both Su(fu) and Fu kinase activity, Ci is now able to enter the nucleus, suggesting that Fu kinase activity is required to regulate the cytoplasmic retention of the Su(fu)-Ci complex (Lefers et al., 2001). It is not known whether Su(fu) or Ci are direct targets of the Fu kinase, and this need not be the case, as modification of other components of the pathway such as Cos2 could allow Su(fu)-Ci complex release.

Role of the NLS in Ci nuclear import

While previous studies demonstrated the functionality of the Ci NLS at AA R596-K600 and K611-K614 (Wang and Holmgren, 1999), the data presented here indicate that Ci nuclear import in salivary glands and in wing discs does not absolutely require the presence of this NLS. Consistent with some decrease in NLS function, Ci-mutNLS gives substantial, but not complete, rescue of a *ci* null mutation. This suggests either the existence of an additional NLS within Ci or the presence of an additional protein that brings Ci into the nucleus.

The results from salivary glands may favor the hypothesis that an additional NLS is present in Ci. Ci-mutNLS nuclear import was impeded but not prevented, implying that if an additional protein were necessary to bring Ci into the nucleus, it too would have to be present in salivary glands and would not be Ci specific. Another consideration is that perhaps the Ci-mutNLS mutation does not entirely destroy NLS function. This mutation only disrupts the second basic cluster within a bipartite NLS, as altering the first cluster would disrupt the last zinc finger and DNA binding.

Addition of an exogenous SV40NLS to Ci led to more rapid nuclear import in salivary glands and a variable gain of function phenotype in embryos. The increased rate of nuclear import appeared to compromise the ability of Su(fu) to sequester Ci-SV40NLS in the cytoplasm of anterior wing imaginal disc clones that are away from Hh signaling. One could interpret this result to suggest that Su(fu) masks the endogenous Ci NLS but not the added SV40NLS. This seems unlikely as wild-type Su(fu)-Ci complexes readily enter the nuclei of salivary glands. An alternative explanation is that away from Hh signaling the Su(fu)-Ci complex has some affinity for a cytoplasmic tether and some low probability of being imported into the nucleus; increasing the rate of nuclear import shifts this equilibrium resulting in nuclear accumulation of Su(fu)-Ci.

Potentially consistent with the role of Su(fu) in sequestering Ci in the cytoplasm is the observation that N-terminally myc-tagged Su(fu) appears to be tightly tethered in the cytoplasm resulting in the cytoplasmic retention of both it and Ci. It is possible that the addition of the myc tag causes a spurious interaction between Su(fu) and an unidentified cytoplasmic component, or it may be the case that the interaction is normal, but the addition of the myc tag prevents the release of Su(fu) from this component. As these experiments were carried out in salivary glands, where there is little if any Fu, Smo or Cos2, the component tethering myc-Su(fu) is distinct from the known proteins in the Hh signal transduction cascade.

Ci-SV40NLS is not sequestered in the cytoplasm of salivary glands by myc-Su(fu). Instead much of it escapes into the nucleus, but it does not bring myc-Su(fu) with it. Again, this result is likely a consequence of the increased rate of Ci-SV40NLS nuclear import. Myc-Su(fu) remains tightly tethered in the cytoplasm, and the distribution of Ci-SV40NLS will be

determined by the relationship between the rate of nuclear import and the binding affinity to myc-Su(fu).

Su(fu)-Ci complex binds DNA target sites

Mammalian studies have found that the addition of mouse Su (fu) can increase the binding affinity of the Glis to target DNA sequences (Pearse et al., 1999). Our salivary gland model system demonstrates that Su(fu), along with Ci, clearly bind an introduced target within the polytene chromosomes. The presence of Su(fu) at Hh target gene enhancers provides the opportunity for another level of Ci regulation. This regulatory role is likely to be restricted to the full-length form of Ci. The Ci repressor form is missing the C-terminal Su(fu) binding site (Croker et al., 2006) and Ci-N[HA]Zn, which closely resembles the Ci repressor, does not bring Su(fu) with it to the DNA.

Dual role for Su(fu) in the regulation of Ci

Given the high degree of conservation between the mammalian and *Drosophila* Hh signaling pathways, one might expect Su(fu) to play homologous roles in the negative regulation of the two pathways. Su(fu) has been shown to act in the cytoplasm as a negative regulator of the pathway by contributing to the sequestration of Ci (Lefers et al., 2001; Methot and Basler, 2000; Wang et al., 2000). In the absence of Hh, Fu and Ci are tethered to microtubules via their interaction with Cos2. In the presence of Hh, Smo is phosphorylated, the complex is released from the microtubules, and a tetrameric cytoplasmic complex is formed with the addition of Su(fu) (Denef et al., 2000; Stegman et al., 2000). Su(fu) may contribute to Ci sequestration in two ways, as part of the tetrameric cytoplasmic complex and as a heterodimer with Ci where it could act as a sink to sequester any excess Ci that is not bound to the Cos2-Fu complex.

The presence of Su(fu) in the nucleus suggests a dual role for Su(fu) in the regulation of Ci (Fig. 8A). A direct nuclear role for Su(fu) negative regulation has been inferred by vertebrate studies documenting an interaction of Su(fu) with SAP18 (Cheng and Bishop, 2002; Paces-Fessy et al., 2004). While mammalian Su(fu) has been show to interact with SAP18 through GST pull-downs and yeast two-hybrid analysis (Cheng and Bishop, 2002; Paces-Fessy et al., 2004), initial yeast two-hybrid studies did not reveal an interaction between *Drosophila* SAP18 and *Drosophila* Su(fu) (Paces-Fessy et al., 2004). Therefore, further studies are needed to delineate the function of *Drosophila* Su(fu) in the nucleus and to determine if SAP18 is indeed involved in the negative regulation of Ci.

The question still remains of how differential responses to Hh signaling are generated. Ho et al. (2005) demonstrated that in the presence of Hh, Su(fu) is phosphorylated and suggests that this modification at the A/P boundary may reduce Su(fu) repressive activity, thus allowing Ci to activate target genes requiring the highest levels of Hh. The modification of Su(fu) or Ci could change the nature of cofactors recruited to target enhancers and account for differential gene regulation.

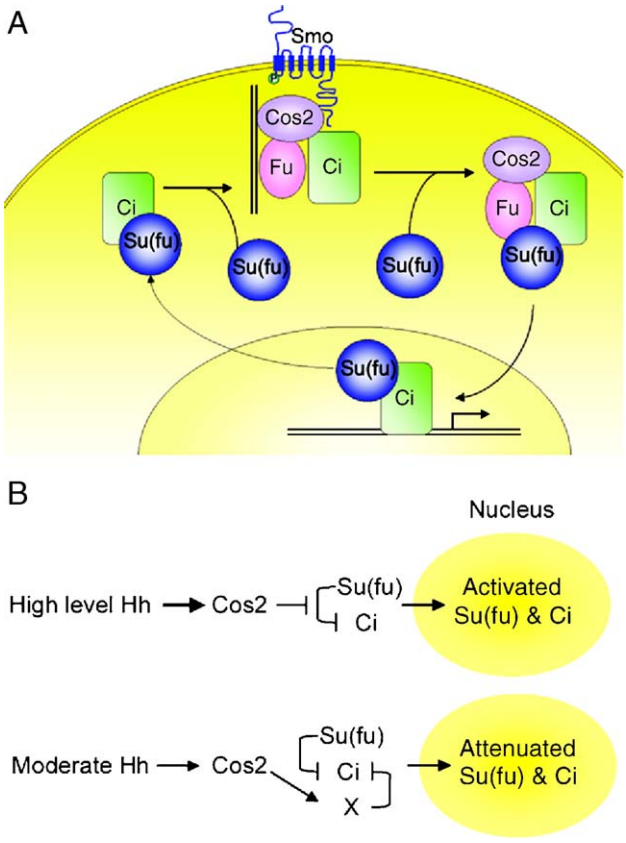

Fig. 8. Model of Ci regulation by Su(fu). (A) Dual mechanisms of Su(fu) mediated Ci regulation. Ci exists in multiple cytoplasmic complexes, a microtubule associated Cos2/Fu/Ci complex, and a tetrameric cytoplasmic complex that is formed with the addition of Su(fu) (Stegman et al., 2000). In this context Su(fu) negatively regulates Ci by contributing to its cytoplasmic sequestration. Additionally, a third complex consisting of just Ci and Su(fu) may also act as a sink to sequester Ci that has not already been bound to the other complexes. Hh signaling leads to the release of the Ci-Su(fu) heterodimer and its translocation to the nucleus where Su(fu) may also play a role in Ci regulation by recruiting transcriptional corepressors. (B) Regulation of Ci activation by Su (fu) and Cos2. In response to high level Hh signaling, Cos2 is required to generate activated Ci, likely by mediating modification of the Ci-Su(fu) heterodimer. In response to more modest levels of Hh, Cos2 does not block Su (fu) attenuation of Ci and contributes to further attenuation by acting on a second component "X".

This model is consistent with observations on the phenotypes of *cos2* and *cos2*; *Su(fu)* double mutant clones. In *cos2* mutant clones, targets that require modest levels of Hh are activated while those that require high-level Hh are not (Wang and Holmgren, 1999). This suggests that the Cos2 protein is required for some modification of the Ci-Su(fu) heterodimer that is essential for "activation." When the *Su(fu)* gene is also eliminated, now target genes requiring high level Hh are activated (Wang et al., 2000). Thus, Su(fu) must contribute to the attenuation of Ci activity in response to modest levels of Hh. However, Su(fu) cannot be the entire story as animals mutant for Su(fu) are essentially normal. There must be a second factor that is partially redundant with Su(fu) in attenuating Ci activity (Fig. 8B). As *cos2*; *Su(fu)* mutant clones have "activated" Ci, it would seem that Cos2 is required for the function of this second factor.

Acknowledgments

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